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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [5]: C12N 15/12, 1/21, C12P 21/02 A61K 37/02, C12P 21/08 G01N 33/68, C07K 13/00	**A1**	**(11) International Publication Number:** **WO 91/05043** **(43) International Publication Date:** 18 April 1991 (18.04.91)

(21) International Application Number: PCT/EP90/01677

(22) International Filing Date: 8 October 1990 (08.10.90)

(30) Priority data:
P 39 33 850.9   6 October 1989 (06.10.89)   DE

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: CYTOLYSIS INHIBITOR PROTEINS (CLI) AND DNA SEQUENCES CODING FOR SAID PROTEINS

(57) Abstract

A cytolysis inhibitor protein, DNA coding therefor, plasmids containing said DNA, host cells containing said plasmids and monoclonal antibodies to said protein are provided, as well as a method of isolating the cytolysis inhibitor for bodily fluid, a method of making the cytolysis inhibitor by recombinant technology, immunological methods of use of said antibodies and methods of treating various disease states.

CYTOLYSIS INHIBITOR PROTEINS (CLI) AND DNA SEQUENCES CODING FOR SAID PROTEINS

Background of the Invention

5   The invention relates to a novel blood plasma component having a strong inhibitory effect on the target cell lysis mediated by terminal complement proteins, such as, for example, by perforin secreted by killer cells or by the α-toxic of Staphylococcus aureus. The component

10   likewise plays possibly an important role in lipid metabolism.

Two effector systems, complementing each other, ensure immunological defense against entering viruses, bacteria, virus-transformed and virus-infested cells,

15   endogenous cells which are altered in malignant fashion or due to ageing, against sperms in the female genital tract, and against exogenous material of the environment.

The cellular effector system consists of specially matured immune cells, the so-called cytotoxic T-lympho-

20   cytes and the natural killer cells. Lymphocytes and natural killer cells are capable, with the aid of inherent surface receptors, of recognizing exogenous structures on other endogenous cells indicative of abnormal states or in intersticial tissue, as well as

25   structures on foreign materials such as, e.g., invading microorganisms, and of effectively preventing the spreading and proliferation of these exogenous materials,

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endogenous cells and microorganism. Activated cytotoxic
lymphocytes and natural killer cells enter into intimate
contact with the target cells recognized as foreign and
release membrane-damaging molecules in a controlled
fashion, the lymphocytes and killer cells themselves
being protected against these molecules. The actual
membrane-damaging molecule of the killer cells is the so-
called perforin possessing high affinity for the
lipophilic membrane of the target cell. Several
molecules of perforin penetrate into the double-layered
membrane of the target cell and thereafter form a
cylinder-like water-permeable channel transversely
through the lipid double membrane of the target cell.
Water molecules and electrolyte salts can freely move
through the hydrophilic inner space of the
transmembranous channel. Thereby the osmotic equilibrium
of the cell is disturbed, and the target cell will die
due to the influx of sodium chloride- and calcium-
containing water from the extracellular environment

     The humoral system of immune defense consists of a
plurality of soluble plasma proteins which interact with
one another and form a finely staggered cascade similar
to that of the clotting system. The actual terminal step
in complement activation leads to membrane damage to the
target cell according to the same principle as in case of
perforin-caused lysis of the target cells. Grouping
together of the terminal components of the complement
system is initiated by the cleavage of complement protein
C5 into two fragments C5a and C5b. The carboxy-terminal
main fragment C5b unites with C6, C7 and C8 to a
macromolecular complex, the hydrophobicity of which
increases considerably with the addition of C7 and C8 and
which can bind to the lipid double membrane. The C5b-8
complex proper does not as yet form a functional pore in
the lipid membrane. Membrane-bound, it takes over the

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part of a receptor controlling the addition of several C9
molecules to form a permeable membrane channel.

Soluble perforin monomers and the stable, soluble
C5b-6 complex can move away from the site of their
5   release and/or generation and can diffuse into the
surroundings so that neighboring healthy cells could be
affected.   Therefore, it has been supposed for a long
time that effective regulatory mechanisms exist which
prevent uncontrolled propagation of potentially lytic
10 molecules.

Summary of the Invention

The present invention provides purified natural
inhibitor for the known immunological effector molecules,
i.e., for perforin stemming from T cells and killer cells
15 and for the terminal complement proteins of human plasma,
as well as inhibitors produced by genetic engineering.

The invention further provides DNA which codes for
the specific protein portion of a novel blood plasma
component and to a purification process for the natural
20 blood plasma component characterized by the CLI protein
molecule and exerting an inhibitory effect on the target
cell lysis mediated by complement proteins and on
perforin isolated from killer cells.   The invention
especially concerns the nucleotide base sequence of the
25 CLI-1 clone depicted in Figure 2 and the amino acid
sequence derived therefrom.   The invention furthermore
provides the plasmid pGEM4/CLI-1 shown in Figure 1; host
organisms transformed with this plasmid; and cytolysis
inhibitor from human plasma isolated for the first time.
30 This invention furthermore provides hybridoma cell
lines, e.g., hybridoma cell line CLI-9, the monoclonal
antibodies, e.g., monoclonal antibody CLI-9 produced by
the hybridoma cell line CLI-9, and the use of said
antibodies for the isolation and purification of CLI from

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human plasma and its use for the quantitative
determination of the natural blood plasma component, of
the polypeptide coded by CLI cDNA, and of the CLI-
associated reaction products in biological fluids.

Finally, this invention also involves the
pharmaceutical utilization of CLI for therapeutic and
prophylactic purposes.

Upon further study of the specification and appended
claims, further objects and advantages of this invention
will become apparent to those skilled in the art.

Various other objects, features and attendant
advantages of the present invention will be more fully
appreciated as the same becomes better understood when
considered in conjunction with the accompanying drawings,
and wherein:

Figure 1 shows a scheme of the recombinant
pGEM4/CLI-1 plasmid containing the entire coding sequence
for the cytolysis inhibitor, and several important
restriction scission sites. The plasmid has an
ampicillin resistance gene coding for the enzyme beta-
lactamase, and includes the Sp6 and, respectively, T7
promoter in the direct vicinity to the cloning scission
site BamHI in the polylinker. The two ends of the cDNA
insertion are provided with the adapter oligomers A and B
according to Haymerle, H., Herz, J., Bressan, G.M.,
Frank, R. and Stanley, K.K., Nucleic Acids Res. 154,
8615-8624 (1986). This plasmid was deposited in E. coli
K12 on March 28, 1989 with the DSM (German Microorganism
Depository) under DSM No. 5269;

Figure 2 shows the nucleotide base sequence of the
CLI-1 clone and the amino acid sequence derived therefrom
in the line therebelow. The amino-terminal sequences of
the a-chain (amino acids 1-205) and the b-chain (206-
427) are underlined. On the left-hand margin of the
figure, the numbering is indicated for the nucleotide

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bases, and on the right-hand margin of the figure, the numbering of the amino acids is set forth;

Figure 3 shows the results of the Southern analysis: the position of the molecular weight markers is indicated in kilobases on the left-hand margin of the figure;

Figure 4a shows the purified CLI after separation by SDS polyacrylamide gel electrophoresis (10% polyacrylamide proportion) under nonreducing (left) and reducing conditions (right);

Figure 4b shows the purified CLI after separation by SDS polyacrylamide gel electrophoresis (12% polyacrylamide proportion);

Figure 5 shows a Western blot identification (bars 1 to 3') of CLI in human plasma (1), in purified C5b-9 complex (2), in seminal fluid (3 and 3'). Bar 4 shows the proteins of the seminal fluid after staining the SDS gel with "Coomassie" blue;

Figure 6 shows the inhibition of complement lysis by CLI: CLI inhibits lysis of the erythrocytes by soluble C5b-6, C7, C8 and C9 in a concentration-dependent way (bottom curve), but does not inhibit lysis of C5b-7 erythrocyte intermediate stages by C8 and C9 (top curve);

Figure 7 shows the inhibition of perforin-mediated erythrocyte lysis at various CLI concentrations;

Figure 8 shows the inhibition of staphylococcus-α-hemolysin mediated erythrocyte lysis by CLI. Staphylococcus-A-hemolysin is preincubated with varying CLI concentrations at 37°C in GVB buffer and thereafter mixed with a rabbit erythrocyte suspension in GVB buffer. Subphysiological CLI concentrations (15μg/ml) almost completely suppress the toxin-caused lysis of the erythrocytes; and

Figure 9 shows that ApoA-I-CLI complexes appear in the electron microscope as spherical structures having a diameter of 9.0 ± 1 nm.

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The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

5   From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and

10   conditions.

It was found during the characterization of the soluble terminal complement complex with the aid of various monoclonal antibodies (which were raised against said complex) that terminal complement complex contains,

15   in addition to the already known S-protein/vitronectin, another still unknown protein. This unknown protein was located by a monoclonal antibody, CLI-9, which, contrary to the expectation, did not recognize the S-protein. It was found that this protein, cytolysis inhibitor (CLI),

20   is not a component of the membrane-bound, lytic complement complex.

By means of affinity chromatography with the aid of the monoclonal antibody CLI-9, according to routine methods, the corresponding component from human plasma

25   was isolated in a functionally active form and an amino acid sequence was derived therefrom using standard protein sequencing technology.

The amino acid sequence of part of the glycoprotein CLI was determined by sequencing the first twenty amino-

30   terminal amino acids in accordance with the Edman degradation method. Synthetic DNA probes were then prepared with the aid of these sequences and used to

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screen a human liver-specific cDNA library, according to
routine methods recited in Example 1. The novel gene was
then cloned from the human genome. This gene codes for a
protein molecule carrying the international name of
5   "Cytolysis Inhibitor" (CLI or ZLI). This polypeptide is
an essential and characteristic part of a novel blood
plasma component with the above-mentioned properties.
        After isolating the recombinant plasmid pGEM4 of the
clone CLI-1 according to a routine method, this plasmid
10   was analyzed with the aid of restriction enzymes. The
size of the DNA insertion in the pGEM4 vector was
determined.
        Purified CLI was found to have a molecular weight of
70 kilodaltons under nonreducing conditions and a
15   molecular weight of 35 kilodaltons under reducing
conditions in sodium dodecyl sulfate gel electrophoresis.
The protein was also shown to be a glycoprotein. A
comparison with all heretofore known sequences published
in the literature showed that the complete protein
20   structure of CLI and the corresponding complete
nucleotide base sequence had not been determined
heretofore.
        The term "CLI" as used herein refers to the
glycosylated and unglycosylated form of the protein,
25   having the biological activity of inhibiting cytolysis
mediated by cytolytic proteins, such as, e.g., complement
or perforin, as well as to all isoforms of CLI which may
differ from the natural glycosylated form found in human
serum by their carbohydrate content. The term also
30   includes therefore the CLI of seminal plasma which may
have other carbohydrate structures.
        Based on the immunological and molecular-biological
identification of the glycoprotein CLI, a novel component
was isolated from human plasma in adequate amounts with
35   the aid of monoclonal antibodies, was purified, and was

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investigated for its inhibitory effect. The isolated
blood plasma component counteracts undesired cellular
destruction. It neutralizes, in a way dependent on
concentration, the cytolytic potential of C5b-7
5   complement complexes in the extracellular fluid and in
the plasma. The sensitivity of endogenous cells to
complement and perforin lysis is diminished, and the
threshold for cellular damage is increased. The
glycoprotein CLI also occurs in human seminal fluid.
10   Intensive studies of the natural CLI blood plasma
component (Example 2f) yielded the following results. In
human blood plasma, CLI is fixedly associated with the
apolipoprotein A-I (ApoA-I), which transports
predominantly phospholipids and free as well as
15   esterified cholesterol. In lipoprotein gel
electrophoresis (Paragon system, Beckman), the ApoA-I-
CLI particle migrates in the pre-beta zone. Since the
ApoA-I concentrations in the plasma are about 20 times
higher than the CLI concentrations, about 2.5% of the
20   entire plasma ApoA-I is present in the form of an ApoA-
I-CLI complex. High-density lipoproteins of the pre-
beta fraction represent, on the average, a proportion of
4.2 ± 1.8% of the entire ApoA-I in the plasma.
Therefore, the predominant proportion or even the entire
25   fraction of pre-beta HDL could consist of ApoA-I-CLI
particles. Thus, the term "natural CLI blood plasma
component" as used herein refers to the entire
apolipoprotein A-I (ApoA-I)/CLI/lipid complex found in
whole human blood. It is further noted that this complex
30   may be heterogeneous, e.g., it may bind additional
components of lipid transport pathways, cholesterol-
ester-transferrin protein (CETP), lecithin cholesteryl
acyltransferase (LCAT) and/or apolipoprotein A-II. The
lipid content of the HDL-CLI complex can vary among

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individuals and even in the same individual after eating
or during fasting.

In earlier works (Castro and Fielding, Biochemistry
27:25-29, 1988), it was demonstrated that cellular
5   cholesterol is primarily taken up by pre-beta HDL and
then is further transported to alpha HDL and LDL.
Consequently, ApoA-I-CLI particles could represent an
important link in the transfer of excess cellular
cholesterol from the cell to LDL and HDL and in the
10   cholesterol supply of peripheral tissues via LDL and HDL
in the reverse direction. The CLI-HDL particles
described by the inventors could be significant in total
for the antiatherogenic effect of HDL.

The natural CLI blood plasma component isolated with
15   the aid of the monoclonal antibody CLI-9 is capable, due
to its special biochemical properties, of neutralizing
the membrane-binding and membrane-inserting activity of
the nascent complement complexes and of the monomeric
perforin. The biological activity of the purified blood
20   plasma component could be mediated by the polypeptide
chain, the carbohydrate proportion associated therewith,
or by CLI-associated lipid proportions.

The anticytolytic activity of CLI was confirmed by
in vitro experiments with sheep erythrocytes. The
25   efficiency of the erythrocyte lysis was hampered with
increasing CLI concentrations. The effective CLI
concentrations are in a range lying in the proximity of
or somewhat below the natural plasma level.

The complete cDNA sequence of CLI was isolated from
30   a liver-specific gene bank and analyzed with the use of
methods of genetic engineering and based on the provided
partial amino acid sequence. It is noted that this
sequence coincided with an already published partial
sequence.

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Southern blot analysis demonstrates that CLI is
represented merely by a single gene in the genome of man
and in rats. The existence of very similar or highly
homologous genes, i.e., the presence of a gene family,
5   was thereby excluded with high probability. This result
is particularly important in view of the fact that it was
possible, with the aid of the monoclonal antibody CLI-9,
to unequivocally identify the cytolysis inhibitor also in
seminal fluid. The molecular weight as well as the
10   reactivity with the monoclonal antibody CLI-9 support the
conclusion that the protein in human seminal fluid
involves the same gene product.

The clone CLI-1 which was isolated as described
herein has a length of 1651 base pairs and exhibits an
15   open reading frame starting with the 199th nucleotide
base. The amino acid sequence derived from the
nucleotide base sequence has 448 amino acid residues.
The first 21 amino acids form a typical hydrophobic
signal peptide which is removed during translocation into
20   the rough endoplasmic reticulum. The mature secreted
protein thus begins with the sequence D-Q-T-V-S-D-N-E
(aspartic acid, D, is number 1). The double-chain form
of CLI is due to the fact that under reducing conditions,
even prior to secretion of CLI into the human plasma, the
25   peptide bond between arginine-205 and serine-206 is
hydrolyzed by a still unknown cellular protease.

The structural properties of CLI are typical of
apolipoproteins. The predicted α-helix content of CLI is
41%. There are several helical segments of amphiphilic
30   nature (residues 1-76, 150-170, 215-240, 300-350 and 406-
420) which are conserved across human, rat and quail
species. Similar amphiphilic structures are shared by
all apolipoproteins, e.g., ApoA-I and A-II. These
predicted secondary structural elements are likely to
35   interact with lipid bilayers and the lipids of the CLI-

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HDL complex. The hydrophobic surface of these protein
segments are expected to mediate contact with the
hydrophobic leaflet of lipid bilayers, the lipid micelles
of CLI-HDL complexes and the hydrophobic, membrane-
inserting domains of channel-forming, cytolytic proteins
of the terminal complement system, the killer cell-
derived lytic protein perforin and cytolytic proteins of
pathogenic microorganisms. The hydrophilic surfaces are
exposed to the surrounding hydrophilic fluid (plasma,
interstitial fluid) and determine the water solubility of
the CLI-HDL complexes.

By "corresponding to" is meant sequences that are
identical to the DNA and amino acid sequences
particularly identified herein or are different due to
natural variations which produce proteins having a
biological activity of CLI. Thus, in addition to the
particular DNA sequence indicated herein, this invention
also encompasses DNA sequences coding for the same
protein but differing due to degeneracy of the DNA code,
as well as sequences coding for variants, e.g., allelic
variants, of the CLI protein which have the biological
activity of CLI, i.e., of inhibiting cytolysis mediated
by cytolytic proteins.

This invention also encompasses DNA sequences coding
for synthetic mutant CLI proteins (muteins) having a
biological activity of CLI. Thus, in addition to the
particular amino acid sequence indicated herein, this
invention also encompasses synthetic variants of the CLI
protein which have the biological activity of inhibiting
cytolysis mediated by cytolytic proteins, as well as such
variants having such activity or which are capable of
binding to antibodies that bind specifically to CLI.
Suitable variants can be recognized, identified and made
by the skilled worker according to the particular use
required by reference to the physical characteristics of

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the natural CLI protein, as outlined above, including,
e.g., the helical conserved regions. Thus, for example,
by replacing moderately hydrophobic residues with more
hydrophobic amino acid residues, the stability and lipid
5   affinity of the CLI-HDL complex can be increased;
conversely, by introducing highly hydrophilic residues
and generating additional asparagine-linked glycosylation
sites (Asn-X-Ser/Thr) at the water interface, the water
solubility and half-life of CLI and CLI-HDL complexes
10   could be increased.

The DNA sequences, plasmids, cytolytic proteins,
antibodies and cell lines of this invention can be
produced by any and all means conventional in the art,
based upon the disclosure of this invention. Specific
15   and general methods for making these products are
described above and below.

Natural CLI can be purified from human bodily fluids
by any means routine to one of ordinary skill in the art,
given the disclosure of this invention. In a preferred
20   method, the monoclonal antibodies to CLI of this
invention are utilized for this purpose by attaching them
to an activated solid support material, e.g., Sepharose,
and contacting a solution containing the bodily fluid,
e.g., plasma or seminal fluid, with the antibody-solid
25   support matrix. The CLI will bind to the antibodies,
while the rest of the constituents of the bodily fluid do
not. The CLI is then obtained in highly purified form
upon release from the antibodies, e.g., by use of a
glycine/NaCl buffer.

30   CLI can also be produced by recombinant DNA
technology according to methods well-known to those of
ordinary skill in the art. Given the amino acid sequence
disclosed herein, as well as the DNA sequence also
disclosed, probes for the CLI gene can be routinely
35   synthesized which are complementary to DNA sequences

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expected to be found in a CLI gene. By using such
probes, DNA sequences for the gene encoding the CLI gene,
e.g., sequences contained in the cloning plasmid
pGEM4/CLI-1, can be isolated from, e.g., endogenous
5   plasmid sequences in genomic libraries, and cloned into
an expression vector suitable for the expression of the
gene, according to techniques which are well-known to one
of ordinary skill in the art.

For uses in which glycosylation of the final protein
10 is not required, the DNA sequence can be expressed in
either non-glycosylating procaryotic or eucaryotic
organisms. For uses in which glycosylation is required,
the DNA sequence may be expressed in organisms, e.g.,
eucaryotic cells, which endogenously perform the required
15 glycosylation. Alternatively, non-glycosylated protein
may be glycosylated in vitro according to well-known
methods, e.g., by use of the appropriate glycosylating
enzymes.

The protein can then be isolated and purified from
20 the cells by means appropriate to the cell type in which
it is expressed; i.e., if produced in a cell type which
secretes the protein, it may be purified from the cell-
free medium separated from the expressing cells. If the
protein remains intracellular, then it can be isolated by
25 any one of numerous means known in the art, e.g., the
cells are mechanically or osmotically ruptured, insoluble
cellular debris is removed from the soluble fraction, and
the desired protein is purified by chromatographic means,
ionic precipitation, HPLC, etc., including the affinity
30 chromatography method also described herein using an
antibody specific for the CLI protein bound to a solid
support.

Antibodies specific for CLI are made by methods well
known to one of ordinary skill in the art, e.g., by
35 multiply injecting purified CLI into mice or rabbits and

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purifying the thus-elicited antibodies from the animals'
serum, e.g., by affinity chromatography using CLI affixed
to a support medium. Monoclonal antibodies are also made
by well-known methods, e.g., according to the well-known
5   method of Kohler and Milstein.

Having antibodies specific to the desired protein in
hand, development of immunoassay tests for the detection
and quantitation of CLI, CLI-fragments, and CLI-
containing complexes, e.g., CLI-ApoA-I and/or CLI-HDL
10   complexes, is a routine matter for one of ordinary skill
in the art. Test development protocols are well-known
and can be modified by routine experimentation to
customize the assay for the detection of CLI. Such
immunoassay tests can be used to detect and establish
15   levels of CLI present in any normal and disease state of
interest in animals, including humans, according to
methods well known to one of ordinary skill in the art.
Thus, for example, serum can then be tested for the
presence of normal/abnormal levels of CLI, either alone
20   or as a complex with various other materials, e.g., with
ApoA-I, or with HDL. Also, seminal fluid can be
routinely tested for the presence of normal/abnormal
levels of CLI. These tests can be routinely performed
and quantitated, e.g., according to well-known methods.
25   Similarly, antibodies according to this invention can be
used to perform immunohistological differential diagnoses
and in vivo diagnoses, e.g., according to the usual
methods known in the art. In particular, for the in vivo
diagnoses, an antibody of this invention can be labelled
30   in a way detectable by external detecting means, e.g.,
radiolabelled for X-ray imaging or labelled with a
paramagnetic species for magnetic resonance imaging, the
labelled antibody administered to the patient, and the
imaging procedure performed.

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CLI-containing complexes, e.g., CLI-ApoA-I and CLI-
HDL, can be isolated from bodily fluids using routine
methods known to one of ordinary skill in the art. For
example, they can be isolated electrophoretically, as
described above, or the anti-CLI-antibody/support
material affinity chromatography method can be employed,
with our without concurrent use of anti-ApoA-I
antibodies. Furthermore, reconstituted complexes
comprising isolated CLI, either purified natural isolated
CLI or CLI produced by recombinant technology, as
described above, can be routinely made in the presence or
absence of lipids, e.g., phospholipids, and optionally
also in the presence of cholesterol, e.g., by mixing the
components in the presences of detergents to totally
solubilize the proteins and lipids (Ann. Rev. Biophys.
Chem. 15, 403-456 (1986)). The detergent is then removed
either by dialysis or molecular sieve chromatography over
a gel filtration column that includes the detergent and
excludes the final reconstituted product. Suitable
detergents include, e.g., sodium cholate, sodium
taurocholate or octylglucoside. Egg phosphatidylcholine
and free cholesterol are commercially available, and
lipid-free ApoA-I can be isolated from human plasma by
the method of Peitsch et al. Analyt. Biochem. 178, 301-
305 (1989).

The cytolytic inhibitor protein of this invention
can be used as a therapy for conditions which are
mediated by cytolytic protein-mediated cell lysis. Thus,
increasing the CLI plasma level, e.g., by administration
of exogenous CLI, represents a therapeutic approach for
counteracting increased systemic complement activity in
the body caused by various disease and traumatic states,
as well as the activity of perforin-induced cell lysis,
and/or activity of other membrane-active cytolytic
proteins.

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Further approaches made possible by this invention
include control of plasma levels of the cytolysis
inhibitor by pharmaceutical or biologically active
compounds altering the gene expression of the cytolysis
5   inhibitor in the liver or in other tissues, e.g., the
Sertoli's cells. Immunological methods for determining
the CLI concentrations in human body fluids are also
encompassing this invention.

CLI has been postulated to have a role in human
10   fertility, in that it is involved in preventing premature
lysis of the cell membrane of the sperm prior to
fertilization of the egg. Therefore, congenital or
acquired lack of CLI may be a cause of male infertility.
Similarly, the presence of CLI-specific antibodies in
15   intravaginal and/or intrauterine fluid may also be a
cause of infertility. Therefore, CLI or fragments
thereof, including the carbohydrate portion of the
glycoprotein, are used to develop a vaccine against CLI,
thereby producing in a male immunized therewith a
20   contraceptive effect. . Such a vaccine is produced and
administered according to well-known methods.

The invention additionally includes the systemic or
local administration of CLI for the treatment of tissue-
destroying pathological conditions evoked by complement
25   and killer cells, and the local or systemic
administration of CLI for detoxification of membrane-
active peptides and cytolytic proteins secreted by
pathogenic organisms, for example by bacteria (inter alia
Staphylococcus aureus, Escherichia coli), fungi, or
30   insects (for example, in the poison of honeybees, wasps,
hornets, bumblebees).

The CLI-protein, glycoprotein, protein fragments
and/or complexes, e.g., CLI-ApoA-I or CLI-HDL, are
administered for the various treatment protocols
35   analogously to other inhibitory proteins, e.g., protease

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inhibitor, which are used in animal, especially human, medicine. Particular dosages for a given indication, patient and compound or complex can be routinely determined, e.g., by using standard pharmaceutical
5   procedures well known to practitioners.

Furthermore, due to the nature of the cytolytic process, and the intimate involvement of CLI in controlling it, the presence of CLI in particular locations in tissues throughout the body (e.g., in above-
10   background amounts) is an indication of various types of cellular damage and conditions. Thus, for example, CLI can be involved in tissue damage resulting from, e.g., chemical, metabolic, hormonal, ischemic, mechanical, and immunological causes, as well as in response to
15   involution of normal human tissues. Furthermore, CLI localization during cytolytic tumor therapy is useful to detect the progress and/or regression of such tumors. Thus, the antibodies to CLI have a particular utility for the immunological detection, quantitation,
20   immunohistological differential diagnosis, and localization of CLI and/or CLI-containing complexes in patients, including in bodily fluids, tissue samples, histological specimens, and, in the case of in vivo imaging as discussed above, in a whole patient, by use of
25   appropriately labelled antibodies, which can be labelled according to any of the many suitable ways known to one of ordinary skill in the art for labelling proteins in general and immunological molecules in particular.

For all applications noted herein, CLI and variants
30   thereof and muteins, as well as the DNA sequences, plasmids and antibodies coding therefor or binding thereto, are similarly useful. Furthermore, muteins, which may or may not have a particular biological activity of CLI, can be used to map the binding sites of
35   monoclonal antibodies and to study the domain interaction

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between ApoA-I and CLI, and for quality control, e.g., of production processes.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description,

5   utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

10   In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius and unless otherwise indicated, all parts and percentages are by weight.

The entire disclosures of all applications, patents

15   and publications, if any, cited above and below, and of corresponding application German P 3933850.9, are hereby incorporated by reference.

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Examples

Example 1

Isolation of the Cytolysis Inhibitor (CLI) Gene
==

      (a)   Designing of a Probe and Production

5   of Synthetic Oligonucleotides

        Nucleotide acid sequences are derived via the
genetic code from the partial amino acid sequence of the
two chains of CLI, and the corresponding oligonucleotides
are synthetized as hybridization probes. Since the

10   genetic code is degenerated, i.e. in most cases several
codons can code for the same amino acid, two long
oligonucleotides are synthetized and, in accordance
with the rules by Lathe, J. Mol. Biol. 183 : 1-12, 1985,
only those codons are selected which, based on statistic-

15   al empirical values, can be found with maximum probability
in human genese for the respective amino acids. The
partial sequence DNELQEMSNQG is selected for probe 1,
and the partial sequence PYEPLNFHAMFQPFLEM is chosen for
probe 2. These protein sequences are determined in

20   accordance with Example 2   and/or according to Murphy
et al., J. Clin, Invest. 81 : 18589-1864, 1988. The
resultant synthetic hybridization probes have the fol-
lowing nucleotide base sequences for probe 1:   5'-GAC
AAT GAG CTG CAG GAG ATG TCC AAC CAG GG-3'; for probe 2:

25   5'-CCC TAT GAG CCC CTG AAC TTC CAC GCC ATG TTC CAG CCC TTC
CTG GAG ATG-3'. Comparison with the subsequently de-
termined, correct cDNA sequence shows that both probes
deviate merely by three nucleotide bases from the correct
cDNA sequence, and probe 1, a 32-oligomer, codes for

30   the amino acids 6-15 of the a-chain and the probe 2, a
51-oligomer, codes for the amino acids 7-23 on the b-
chain.

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(b)   Terminal Marking of the Oligomer Probes

The probes are labeled radioactively on the
5'-terminus with the use of [gamma-^{32}P]dATP (Amersham)
and T4 polynucleotide kinase (Pharmacia-LKB, Sweden).
5   The 20 µl reaction solution contains 70 mmol of tris-
hydrochloride, pH 7.6, 10 mmol of $MgCl_2$ and 5 mmol of
DTT, 3 pmol of the respective oligomer, 9 pmol of
[gamma-^{32}P]dATP, and 6 units of T4 DNA polynucleotide
kinase, and is incubated for 30 minutes at 37° C. The
10   free, nonused [gamma-^{32}P]dATP is separated from the
oligonucleotides by gel filtration (NAP 25, Pharmacia-
LKB, Sweden).

(c)   Screening of a cDNA Library of the Liver

.The so-called colony hybridization for bacterial
15   clones is performed with the use of large colony densi-
ties. Approximately 200,000 bacteria of a liver-
specific cDNA library (Haefliger, J.-A., Jenne, D.,
Stanley, K.K., and Tschopp, J.: Biochem. Biophys. Res.
Commun. 149 : 750-754, 1987) are spread onto 20
20   ampicillin-containing (50 µg/ml) agar plates having a
size of 22 cm. x 22 cm. The agar medium employed is
10 g/l of "Bacto-Trypton", 5 g/l of NaCl and 1.6% (wt./vol.)
"BactoAgar". After cultivating the bacteria for 20 hours
overnight at 37° C, the roundish colonies of a size of
25   about 1 mm are transferred by simple kiss method from
the agar plates to nitrocellulose filters (Schleicher
und Schüll). The bacterial colonies are further
cultivated on the original agar substrates for 7 hours
and in this way a replicate capable of growth is
30   provided for each colony on the nitrocellulose filters.

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The bacterial colonies on the nitrocellulose filters
are solubilized by means of sodium dodecyl sulfate
and sodium hydroxide solution. During incubation of the
nitrocellulose filters in an alkaline solution, the
5   bacterial DNA is released, denatured, and bound as
single-strand DNA to the filter membrane. After
several washing steps with neutral buffers, the DNA
is firmly fixed to the nitrocellulose by heating
(Davies, L.G., Dibner, M.D., and Battey, J.F.,
10   Basic Methods in Molecular Biology, Elsevier, New
York, 1986). In order to prevent unspecific binding
of the probe to the nitrocellulose, the filters are pre-
hybridized for four hours at 50° C. The solution consists
of 6 x SSC (1 x SSC contains 0.15 moles NaCl, 0.015
15   moles sodium citrate), 5 x Denhardt's solution (according
to Maniatis, T., et al. in Molecular Cloning, Cold
Spring Harbor Laboratory, New York, 1982), 0.1% sodium
dodecyl sulfate, 10 mmol EDTA and 50 µg/ml salmon sperm
DNA (= hybridization solution) subjected to ultrasound
20   and denatured by boiling for five minutes. Hybridization
with the 51 oligomer probe takes place at 50° C in
fresh hybridization solution over a period of four hours.
The concentration of the radioactive probe in the
hybridization solution is 5 x 10^4 cpm/ml. The nitro-
25   cellulose filters are washed at 45° C in 1 x SSC and
0.1% SDS (weight/volume) for 60 minutes and exposed
overnight to an X-ray film at -70° C. On the next day,
the 51 probe is removed by boiling for 10 minutes in
a 10 mmol EDTA and 1% aqueous glycerol solution. The
30   filters are subjected to a second screening procedure
with the 32 probe. A strong unequivocal signal with
both probes is observed only on three places on the
20 filters. Bacterial clones from these sites are

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examined a second time by streaking and renewed cultiva-
tion on small agar plates. During this process, the
bacterial density is reduced to such an extent that the
colonies stand individually and are not intermingled
5   with neighboring colonies. The analysis of respect-
ively 30 colonies from the three positive regions ·
finally results in 3 independent bacterial clones
reacting with both probes.

          (d)   Characterization and Isolation of the
10   cDNA of the CLI-1 Clone

          The recombinant plasmid of the CLI-1 clone
is isolated according to a routine method and analyzed
by restriction enzymes (L.G. Davies et al., Basic
Methods in Molecular Biology, Elsevier, New York, 1986).
15   The DNA is cleaved with the restriction enzymes BamHI and
KpnI. The resultant DNA fragments are separated by
agarose gel electrophoresis, and the size of the DNA
insertion in the pGEM4 vector is determined. The
length of the cDNA amounts to 1.7 kb; there are no
20   internal BamHI and KpnI scission sites (Figure 1).
Approximately 20 µg of the cDNA is separated from
the pGEM4 vector plasmid by agarose gel electrophoresis
and purified for DNA sequence determination.

          (e)   cDNA Base Sequence Determination
                 ----

25   The cDNA of the CLI-1 clone is separated, after
spontaneous ligation, by ultrasonic treatment in an
ultrasonic water bath into randomly distributed subfrag-
ments and fractionated according to size by electro-
phoresis in 1.3% strength NA-agarose (Pharmacia).
.30 Those fragments which fall within the molecular weight

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range from 300 to 600 bp are isolated, and the ends are
repaired with the aid of T4 DNA polymerase so that smooth
termini are produced. The cDNA fragments produced in
this way are inserted into the SmaI restriction scission
5   site of Ml3mp8 vector with the use of T4 DNA ligase.
The single-stranded phages of the M13 vector are
prepared according to standard methods (L.G. Davies et
al., Basic Methods in Molecular Biology, Elsevier,
New York, 1986), and the nucleotide sequence of about
10   50 subfragments is determined with the aid of the
"Sequenase" kit (United States Biochemical Corpora-
tion). The nucleotide base sequence of the CLI-1 cDNA
is determined at least once on both strands. The over-
lapping partial sequences are compared with the aid of
15   a microcomputer and composed into the total sequence
(Figure 2).

(f)   Southern Analysis of the Genome of
       Rat and Man
       ----

             5 µg of whole DNA of man and of rat (Genofit,
20   Geneva) are separated into fragments with the aid of the
restriction enzymes BamHI, BglII, EcoRI and HindIII and
then segregated electrophoretically in 0.8% agarose.
After transferring onto a nylon mesh membrane by vacuum
blot method (Pharmacia-LKB), the filter membrane is analyzed
25   with the aid of radioactively labeled single-strand cDNA
of the CLI-1 clone. The cDNA probe hybridizes with one
to at most three fragments in the rat and human genome
depending on which of the five restriction enzymes is
utilized (Figure 3). The fact that it is possible to
30   localize the complete cDNA sequence of CLI on a single
restriction fragment in case of the rat (EcoRI) as well

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as in man (BamHI), proves that there is only a single
gene for the biosynthesis of the cytolysis inhibitor
in rat and in man.

Example 2
----

5   Purification, Biochemical Characterization and
     Identification of CLI with the Aid of Monoclonal
     Antibodies
     ===

                    (a)   Isolation of the CLI and S-Protein
                          from Complement-Activated Serum
                          ----

10   The alternative route of complement cascade
     is activated in 1 liter of human serum by the addition
     of insulin (10 mg/ml, Merck). The thus-produced
     soluble terminal complement complex, the so-called
     "SC5b-9" complex, is isolated according to Bhakdi and
15   Roth (J. Immunol., 127 : 576-582, 1981). In case of
     absence of target cell membranes during complement
     activation, this complex is produced by the uniting of
     the complement proteins C5b, C6, C7, C8, C9, S-protein,
     and CLI. In the presence of anionic detergents,
20   S-protein and CLI will dissociate from the complex.
     250 mmol of deoxycholate (DOC) in the solid form is
     added to a solution of 0.6 mg to 1.2 mg/ml of SC5b-9
     complex. This solution is incubated at 37° C for one
     hour in the presence of 2 mmol of PMSF. Respectively
25   4 ml is loaded onto a linear sucrose gradient (40 ml
     total volume, 10% by weight to 40% by weight of sucrose
     in 6.25 mmol of DOC, 10 mmol of tris-hydrochloride,
     pH 8.1, 50 mmol of NaCl and 7.5 mmol of NaN_3), and
     centrifuged for 3 hours at 4° C and 250,000 g's in a

Beckman vertical rotor (model Vto-50). 2 ml fractions
are collected from the bottom of the centrifuge tube.
The fractions containing S-protein and CLI are pooled
and concentrated approximately fivefold (Amicon PM10

5   membranes). The proteins are then separated with the
aid of a gel filtration column ("Sephacryl" S-300,.
1 cm x 60 cm column, Pharmacia). The equilibration
buffer is 10 mmol of tris-hydrochloride, 50 mmol of
NaCl, pH 8.1. CLI and S-protein elute in a peak.

10   The fractions are concentrated (protein concentration
between 0.4 mg/ml and 0.6 mg/ml) and stored at -20° C.

     (b)   Preparation of Monoclonal Antibodies
           ----

         500 µl of the mixture of S-protein and CLI are
injected subcutaneously into female Balb/c mice in

15   complete Freund's adjuvant (1:1; vol./vol.). After
5 weeks, the injection of 500 µl of the antigen is
repeated, this time in incomplete Freund's adjuvant.
After another 5 weeks, 500 µl of antigen without
adjuvant is administered intraperitoneally, and the

20   mice are then sacrificed after 3-4 days.
         Myeloma cells are cultivated in RPMI 1640
medium containing additionally 10% inactivated fetal
calf serum, 1% glutamine, 5,000 U/ml of penicillin,
5 µg/ml of streptomycin, and 0.02 mmol of 2-mercapto-

25   ethanol (myeloma medium). The myeloma cells are
cultivated at 37° C in 12 Petri dishes having a dia-
meter of 9 cm until they begin to become confluent.
The myeloma cells and the cells from the spleen of
an immunized mouse are mixed with each other in a

30   serum-free medium and pelletized at 1,200 rpm.
The cell sediment is gently resuspended in 2 ml of

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the fusion solution (50% PEG 4000 in RPMI 1640) and
mixed for 2 minutes at 37° C gradually by means of a
pipette. The fusion solution is then diluted with
20 ml of RPMI 1640. Thereafter the cells are sedimented
5   and distributed in 20 ml of fresh myeloma medium on
4 microtiter plates (96 chambers). The culture chambers
contain macrophages obtained by flushing the peritoneal
cavity of 2 mice. On each subsequent second day, the
myeloma medium which, for the purpose of selection,
10 then contains additionally 0.1×10^{-2} mmol of hypo-
xanthine, 4×10^{-4} mmol of aminopterin, and 1.6×10^{-2}
mmol of thymidine (-HAT medium), is changed. Hybridoma
culture supernatants are examined with the aid of an
ELISA test for CLI-and S-protein-specific monoclonal
15 antibodies. Positive hybridoma cells are cloned 3x
and subsequently injected into Balb/c mice pretreated
with 500 ml of pristane. Ascites fluid is removed by
puncture from the abdominal cavity 2-3 weeks later
(Jenne, D., Hugo, F., and Bhakdi, S., Biosci. Rep. 5 :
20 343-352, 1985). The monoclonal antibody, CLI-9, is
not directed toward the S-protein and is utilized for
affinity purification of CLI (Bhakdi, S., Jenne, D., Hugo,
F., J. Immunol. Meth. 80 : 25-32, 1985). By immuniza-
tion with purified CLI, further immunoglobulin-secreting
25 hybridoma lines are established. The hybridoma line
with the designation of CLI-9 was deposited with the
European Collection of Animal Cell Cultures, Salisbury,
SP4 0JG, Great Britain, under "provisional accession
number" 89 052 602. The monoclonal antibodies secreted
30 by CLI-9 react with reduced and unreduced CLI in the
Western Blot analogously as in Example 2(e).

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(c)   Affinity Purification of CLI

        The monoclonal antibody CLI-9 is purified from
1 ml of ascites fluid. For this purpose, the ascites
fluid is passed over a 2 ml protein A "Sepharose"
5   column equilibrated in 10 mmol of tris-hydrochloride,
pH 7.4, 150 mmol NaCl (subsequently called TBS). The
antibodies are eluted with 10 ml of 0.2-molar glycine,
pH 2.8, 0.5-molar NaCl. 4 mg of the purified antibody
is subsequently coupled to 1.5 ml of cyanogen bromide-
10   activated "Sepharose" (in accordance with the directions
by the manufacturer, Pharmacia). For a second column,
20 mg of human immunoglobulin ("Sandoglobin", Red Cross,
Berne) is coupled to 4 ml of cyanogen bromide-activated
"Sepharose". 20 ml of fresh human EDTA plasma (10 mmol
15   EDTA) is diluted with 10 ml of TBS and first applied to
the human IgG affinity column (equilibrated in TBS). The
unabsorbed proteins are thereafter passed through the
anti-CLI affinity column. This column is washed with
10 ml of TBS buffer to which 1.5-moles NaCl has been
20   admixed. Pure CLI is eluted with 10 ml of 0.2-moles
glycine buffer, 0.5 moles NaCl, pH 2.8 (Figure 4).
Approximately 0.5 mg of CLI can thus be obtained in a
buffer volume of 5 ml. Purified CLI is dialyzed against
TBS (16 hours) and either stored at 4° C for several
25   days or deep-frozen (-20° C) in small portions (100 µl).

(d)   Amino Acid Sequence of CLI
----

        10 µg of the purified CLI are dialyzed against
10 mmol ammonium acetate, pH 8.0, and applied dropwise
to a glass fiber membrane treated with "Polybrene".
30   The individual amino acids are degraded stepwise in
accordance with the method by Edman and analyzed with
the aid of an HPLC unit (Applied Biosystems).

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(e)   Detection of CLI in Plasma and in
      Seminal Fluid

The so-called "Western Blot" analysis is especial-
ly well suited for identification of CLI. In this process,
1 µl of the 1:10 diluted seminal fluid or 10 µl of a 1:10
diluted serum solution is separated by SDS-polyacrylamide
gel electrophoresis (Laemmli, Nature 227 : 680-685,
1970) according to molecular weights. The proteins
are subsequently transferred by electrophoresis to
nitrocellulose membranes (Schleicher and Schüll,
15 cm x 10 cm) (Towbin, H., Staehelin, T., and Gordon, J.,
Proc. Natl. Acad. Sci. USA 76 : 4350-4355, 1979). The
electrophoresis buffer consists of 25 mmol tris-
hydrochloride, 192 mmol glycine and 20 vol-% methanol.
The proteins are transferred for 3 hours at 60 V and
220 mA. The nitrocellulose membranes are incubated for
one hour in saturation buffer (1% gelatin [Merck], 0.1%
bovine albumin [Böhringer], 20 mmol tris-hydrochloride,
pH 8.4, 150 mmol NaCl, 5 mmol EDTA and 0.02% sodium
azide), washed three times with TBS, and incubated
overnight with ascites fluid of the monoclonal antibody
CLI-9 (1:1000 dilution) in incibating buffer (= satura-
tion buffer without bovine albumin). After washing three
times in TBS (respectively 5 minutes), either a protein
A peroxidase conjugate (dilution 1:1000, Sigma) or an
anti-mouse IgG peroxidase conjugate (dilution 1:1000,
Dakopatis) is added thereto. The nitrocellulose mem-
branes are incubated for another two hours. The
peroxidase activity is determined with 4-chloro-1-
naphthol (Merck, 0.075%) in the presence of 0.01%
(vol./vol.) of H_2O_2 in a buffer mixture consisting of
20 vol-% methanol and 80 vol-% 10 mmol aqueous tris-HCl,

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pH 6.8. CLI can be clearly identified as a band in
the molecular weight range of 70,000 daltons. The
antibody CLI-9 reacts with unreduced human CLI, but not
with reduced CLI or with CLI from other animal species.

5   (f) Biochemical Characterization of the ·
                    Blood Plasma Component Obtained by
                    Affinity Chromatography with the
                    CLI-9 Antibody

         When examining the various elution fractions
10   of the anti-CLI affinity column (Example 2c) with the
     aid of SDS polyacrylamide gel electrophoresis (SDS-PAGE),
     with a high polyacrylamide proportion (Figure 4b), then
     it can be seen that a further protein having a molecular
     weight of 28 kilodaltons is regularly isolated with CLI as
15   a constant component. After SDS polyacrylamide gel
     electrophoresis under nonreducing conditions, the 28
     kilodalton component was blotted onto an "Immobilon"
     membrane and added to the sequence on the amino-
     terminal end in accordance with the method by Edman
20   (see Example 2d). The frist ten amino acids of the
     28 kilodalton component are Asp-Glu-Pro-Pro-Gln-Ser-Pro-
     Trp-Asp-Arg and exactly coincide with the amino-terminal
     sequence of the apolipoprotein A-I (ApoA-I). The
     ApoA-I of the CLI complex shows the same mobility in
25   the SDS-PAGE as ApoA-I of purified HDL and reacts
     with commerically available ApoA-I-specific antisera
     (Behringwerke, Marburg). From this, the inventors
     have drawn the conclusion that the 28 kilodalton com-
     ponent represents the mature form of the intact ApoA-I.

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Since the same molecular weight form of ApoA-I
is considered to be the specific protein marker of the
high-density lipoproteins (HDL), the supposition presented
itself that the ApoA-I-CLI complex could involve a new
5   special HDL subfraction. Therefore, the inventors
analyzed the lipid content and the lipid composition
of the ApoA-I-CLI complex. The ApoA-I-CLI particle
consists of 78% by weight of protein and 22% by weight
of lipid. The lipids were separated by thin-layer
10   chromatography into individual lipid fractions and
quantified by comparison with commercially available
lipid standards in situ on the thin-layer plate. The
lipid proportion in mol-% is broken down as follows:

        54%   total cholesterol (unesterified to
15   esterified cholesterol, 0.58)

        42%   phospholipids (85% phosphatidylcholine,
                9% sphingomyelin, 6% phosphatidylethanol-
                amine, and other phospholipids)

        4%   triglycerides.

20   Thus, the significant characteristic of the
ApoA-I-CLI lipid particle is the very high protein pro-
portion and the proportion of unesterified cholesterol
which is high in comparison with esterified cholesterol.
Based on the low lipid proportion of 22%, the ApoA-I-
25   CLI lipid particle is not found in the classical
potassium bromide density fraction of HDL (d = 1.063,
1.21 g/ml).

A further indication that the purified ApoA-I-
CLI complex involves an HDL particle is constituted by
30   the results of investigations with the electron micro-
scope. ApoA-I-CLI complexes are presented in the electron
microscope as spherical, homogeneous-appearing structures
of uniform size (Figure 9). The average particle dia-
meter amounts to 9.0±1.0 nm and thus is similar to the

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HDL particles of size category 3 (8.5 nm to 9.6 nm),
the smallest of the three size categories.

Example 3

Biological Activity of CLI
============================

5   (a)   Inhibition of Complement-Mediated Lysis
                   by CLI

             The functional, inhibiting properties of the
natural CLI plasma component, as purified from plasma in
accordance with Example 2(c) will be examined below
10   in the complement lysis test. For the lysis test,
sheep erythrocytes (Biomerieux, 1 x 10^9 cells per ml)
are utilized. The complementary proteins C5b-6
(Podack, E.R., and Müller-Eberhard, H.-J., J. Immunol.
124 : 332-336, 1980), C7 (Podack, E.R., Kolb, W.P.,
15   Esser, A.F., and Müller-Eberhard, H.-J., J. Immunol.
123 : 1071-1077, 1979), C8 (Kolb, W.P. and Müller-
Eberhard, H.-J., J. Exp. Med. 143 : 1131-1139, 1976)
and C9 (Podack, E.R., Tschopp, J. and Müller-Eberhard,
H.-J., J. Exp. Med. 156 : 268-282) are purified in
20   accordance with standard methods. One µg of C5b-6
which lyses, in the absence of CLI, about 80% of the
erythrocytes under the conditions set forth below,
is mixed with 30 µl of sheep erythrocytes in 10 mmol
veronal buffer, pH 7.4, 142 mmol NaCl, 0.1% gelatin
25   and 10 mmol EDTA (abbreviated GVBE), and incubated for
20 minutes at 25° C. Then C7 (1 µg/ml final concentra-
tion), C8 (0.2 µg/ml final concentration) and C9
(1 µg/ml final concentration), as well as CLI in various
concentrations in a range from 0.25 µg/ml to 50 µg/ml
30   are added thereto in a volume of, in total, 30 µl.

Prior to addition, the four proteins had been pre-
incubated for 5 minutes at room temperature. After
an incubation of 30 minutes at 37° C, the intact
erythrocytes are removed by centrifuging (1500 rpm,
5   2 minutes), and the amount of hemoglobin released into
the supernatant is determined at a wavelength of
412 nm in a spectrophotometer.

(b)   Inhibition of Perforin-Mediated Lysis
by CLI
----

10   Perforin is purified according to the method
by Masson, D., and Tschopp, J., J. Biol. Chem. 260 :
9069-9072, 1985. First of all, 30 µl of sheet erythro-
cytes (1 x 10^9 cells/ml) is preincubated with 30 µl of
CLI (various concentrations) for 10 minutes at room
15   temperature. Perforin, which is diluted in GVBE to such
an extent that 80% of the erythrocytes is lysed during
the test, is admixed thereto in 30 µl of buffer volume.
After 30 minutes of incubation at 37° C, the extent of
erythrocyte lysis is determined, as described above in
20   connection with the complement lysis test.

(c)   Inhibition of Staphylococcus-α-Hemolysin-
Mediated Lysis by CLI

Staphylococcus aureus is among the most
frequent bacterial pathogens in man. The α-hemolysin
25   formed by almost all human-pathogenic Staphylococcus
aureus strains is presently considered to be an
important pathogenicity factor. S. aureus secretes
α-hemolysin as a water-soluble monomeric protein with
a molecular weight of 34,000. On the plasma membrane of
30   eucaryotic target cells, respectively 6 toxin monomers
group together into a hydrophilic, transmembranous

channel having a diameter of 1-2 mm. Thereby, the
ionic environment in the interior of the cell is
disturbed; a rapid influx of calcium occurs from the
extracellular space, and, in the extreme case, a
5   colloid-osmotically caused lysis of the target cell
takes place. In sublytic concentrations, the
arachidonic acid cascade can be activated with release
of highly active prostaglandins and leukotrienes in
endothelial cells. Human blood platelets are
10   particularly sensitive with respect to subcytolytic
doses of staphylococcus-α-hemolysin. Due to the action
of the toxin on platelets, platelet aggregation occurs,
along with reduction of the clotting time in the
platelet-rich plasma (Bhakdi et al., J. Exp. Med.,
15   168 : 527-542, 1988).

        Previously, it has been assumed that specific
neutralizing antibodies in the blood plasma and
β-lipoproteins (plasma LDL) represent the only factors
capable of inactivating the staphylococcus-α-hemolysin
20   molecules.

        In order to confirm the biological activity of
CLI with respect to staphylococcus-α-hemolysin,
freshly washed rabbit erythrocytes are utilized in
the test system described below. Staphylococcus-α-
25   hemolysin (staphylolysin reagent, 5 units per bottle,
Behringwerke, Marburg) is diluted in GVB buffer (GVBE
buffer without EDTA, see above) and preincubated at
37° C for one-half hour. Thereafter, 50 μl of a rabbit
erythrocyte suspension (10^8/ml) is admixed to 200 μl
30   of this solution. After incubating for one-half hour
at 37° C, the specimens are centrifuged (2 minutes at
12,000 g's), and the released hemoglobin in the super-
natant is measured by spectrophotometry at 412 nm.
Total hemolysis is measured after adding sodium dodecyl

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sulfate (final concentration, 0.2% by weight). A 1:400
dilution of a working solution with 0.04 unit/ml results
in a 71% hemolysis. This toxin dilution (100 µunits/ml)
is preincubated with various CLI concentrations for one-
half hour at 37° C, then 50 µl of the rabbit erythrocyte
suspension is added, and, after another 30 minutes at
37° C, the hemolysis is measured as above in the various
batches. Figure 8 shows the measuring results of these
tests. Even below physiological concentration (normal
range: 50 - 100 µg/ml), at approxiamtely 15 µg CLI/ml,
the activity of the staphylococcus-α-hemolysin is almost
entirely suppressed.

The preceding examples can be repeated with similar
success by substituting the generically or specifically
described reactants and/or operating conditions of this
invention for those used in the preceding examples.

From the foregoing description, one skilled in the
art can easily ascertain the essential characteristics of
this invention, and without departing from the spirit and
scope thereof, can make various changes and modifications
of the invention to adapt it to various usages and
conditions.

SUBSTITUTE SHEET

Figure 1 shows a scheme of the recombinant
pGEM4/CLI-1 plasmid containing the entire coding
sequence for the cytolysis inhibitor, and several
important restriction scission sites. The plasmid
5   has an ampicillin resistance gene coding for the enzyme
·beta-lactamase, and includes the Sp6 and, respectively,
T7 promoter in the direct vicinity to the cloning
scission site BamHI in the polylinker. The two ends of
the cDNA insertion are provided with the adapter oligo-
10   mers A and B according to Haymerle, H., Herz., J.,
Bressan, G.M., Frank, R. and Stanley, K.K., Nucleic
Acids Res. 154 : 8615-8624, 1986. This plasmid was
deposited in E. coli K12 on March 28, 1989 with DSM
under DSM No. 5269.

15   Figure 2 shows the nucleotide base sequence
of the CLI-1 clone and the amino acid sequence derived
therefrom in the line therebelow. The amino-terminal
sequences of the a-chain (amino acids 1 to 205) and the
b-chain (amino acids 206 to 427) are underlined. On the
20   left-hand margin of the figure, the numbering is indicated
for the nucleotide bases, and on the right-hand margin
of the figure the numbering of the amino acids is
set forth.

             Figure 3 shows the results of the Southern
25   analysis: the position of the molecular weight
markers is indicated in kilobases on the left-hand
margin of the figure.

             Figure 4a shows the purified CLI after sep-
aration by SDS polyacrylamide gel electrophoresis
30   (10% polyacrylamide proportion) under nonreducing (left)
and reducing conditions (right).

             Figure 4b shows the purified CLI after sep-
aration by SDS polyacrylamide gel electrophoresis
(12% polyacrylamide proportion).

Figure 5: Western Blot identification (bars 1 to 3') of CLI in human plasma (1), in purified SC5b-9 complex (2), in seminal fluid (3 and 3').
Bar 4 shows the proteins of the seminal fluid after staining the SDS gel with "Commassie" blue.

Figure 6: Inhibition of complement lysis by CLI: CLI inhibits lysis of the erythrocytes by soluble C5b-6, C7, C8 and C9 in concentration-dependent way (bottom curve), but does not inhibit lysis of C5b-7 erythrocyte intermediate stages by C8 and C9 (top curve).

Figure 7: Inhibition of perforin-mediated erythrocyte lysis at various CLI concentrations.

Figure 8: Inhibition of staphylococcus-α-hemolysin-mediated erythrocyte lysis by CLI.
Staphylococcus-α-hemolysin is preincubated with varying CLI concentrations at 37° C in GVB buffer and thereafter mixed with a rabbit erythrocyte suspension in GVB buffer. Subphysiological CLI concentrations (15 µg/ml) almost completely suppress the toxin-caused lysis of the erythrocytes.

Figure 9: ApoA-I-CLI complexes appear in the electron microscope as spherical structures having a diameter of 9.0±1 nm.

<u>WHAT IS CLAIMED IS</u>:

1.   An isolated DNA sequence coding for a blood
plasma component having a biological activity of
inhibiting cytolysis mediated by a cytolytic protein.

2.   A DNA sequence of claim 1, wherein the
cytolytic protein is perforin, complement protein, or
α-hemolysin.

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3.   A DNA sequence of claim 1, corresponding to the
sequence

CTGCGAACCCTCTCTACTCTCCGAAGGGAATTGTCCTTCCTGGCTTCCAC
TACTTCCACCCCTGAATGCACAGGCAGCCCGGCCCAAGTCTCCCACTA
GGGATGCAGATGGATTCGGTGTGAAGGGCTGGCTGCTGTTGCCTCCGGCTCT
TGAAAGTCAAGTTCAGAGGCGTGCAAAGACTCCAGAATTGGAGGCATG

ATGAAGACTCTGCTGCTGTTTGTGGGGCTGCTGCTGACCTGGGAGAGTGGGC

AGGTCCTGGGGGACCAGACGGTCTCAGACAATGAGCTCCAGGAAATGT

CCAATCAGGGAAGTAAGTACGTCAATAAGGAAATTCAAAATGCTGTCAACGG

GGTGAAACAGATAAAGACTCTCATAGAAAAAACAAACGAAGAGCGCAA

GACACTGCTCAGCAACCTAGAAGAAGCCAAGAAGAAGAAAGAGGATGCCCTA

AATGAGACCAGGGAATCAGAGACAAAGCTGAAGGAGCTCCCAGGAGTG

TGCAATGAGACCATGATGGCCCTCTGGGAAGAGTGTAAGCCCTGCCTGAAAC

AGACCTGCATGAAGTTCTACGCACGCGTCTGCAGAAGTGGCTCAGGCC

TGGTTGGCCGCCAGCTTGAGGAGTTCCTGAACCAGAGCTCGCCCTTCTACTTC

TGGATGAATGGTGACCGCATCGACTCCCTGCTGGAGAACGACCGGCA

GCAGACGCACATGCTGGATGTCATGCAGGACCACTTCAGCCGCGCGTCCAGC

ATCATAGACGAGCTCTTCCAGGACAGGTTCTTCACCCGGGAGCCCCAG

GATACCTACCACTACCTGCCCTTCAGCCTGCCCCACCGGAGGCCTCACTTC

TTCTTTCCCAAGTCCCGCATCGTCCGCAGCTTGATGCCCTTCTCTCCGT

ACGAGCCCCTGAACTTCCACGCCATGTTCCAGCCCTTCCTTGAGATGATAC

ACGAGGCTCAGCAGGCCATGGACATCCACTTCCACAGCCCGGCCTTCCA

GCACCCGCCAACAGAATTCATACGAGAAGGCGACGATGACCGGACTGTGTG

CCGGGAGATCCGCCACAACTCCACGGGCTGCCTGCGGATGAAGGACCAG

TGTGACAAGTGCCGGGAGATCTTGTCTGTGGACTGTTCCACCAACAACCCC

TCCCAGGCTAAGCTGCGGCGGGAGCTCGACGAATCCCTCCAGGTCGCTG

AGAGGTTGACCAGGAAATATAACGAGCTGCTAAAGTCCTACCAGTGGAAGA

TGCTCAACACCTCCTCCTTGCTGGAGCAGCTGAACGAGCAGTTTAACTG

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GGTGTCCCGGCTGGCAAACCTCACGCAAGGCGAAGACCAGTACTATCTGCG

GGTCACCACGGTGGCTTCCCACACTTCTGACTCGGACGTTCCTTCCGGT

GTCACTGAGGTGGTCGTGAAGCTCTTTGACTCTGATCCCATCACTGTGACG

GTCCCTGTAGAAGTCTCCAGGAAGAACCCTAAATTTATGGAGACCGTGG

CGGAGAAAGCGCTGCAGGAATACCGCAAAAAGCACCGGGAGGAGTGAGATG

TGGATGTTGCTTTTGCACCTACGGGGGCATCTGAGTCCAGCTCCCCCCA

AGATGAGCTGCAGCCCCCCAGAGAGAGCTCTGCACGTCACCAAGTAACCAGGC

4.   A DNA sequence of claim 3 having an open
reading frame beginning at the sequence corresponding to
nucleotide 199.

5.   A DNA sequence of claim 4, wherein, upon
expression of said sequence in a host cell, a 21 amino
acid hydrophobic signal peptide coded for by the first 63
bases of the open reading frame is split off from the
remainder of the amino acid sequence during translocation
into the endoplasmic reticulum.

6.   A DNA sequence of claim 3, which is an allelic
variant of the specified sequence.

7.   A DNA sequence of claim 3, which is a sequence
differing from the specified sequence by degenerate
codons.

8.   A vector comprising a DNA sequence of claim 1.

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9.   A vector comprising a DNA sequence of claim 3.

10.   Plasmid pGEM4/CLI-1, deposited in E. coli as DSM No. 5269, a vector of claim 8.

11.   A vector plasmid of claim 8 which is expressible in eucaryotes.

12.   A host organism, transformed with a vector plasmid of claim 8.

13.   A host organism, transformed with the vector plasmid of claim 10.

14.   E. coli, transformed with the vector plasmid of claim 10.

15.   An isolated cytolysis inhibitor protein having the biological activity of inhibiting cell lysis mediated by a cytolytic protein.

16.   A cytolysis inhibitor of claim 15, wherein the cell lysis is mediated by a complement protein, perforin, or α-hemoglobin.

17.   A cytolysis inhibitor of claim 15, corresponding to the amino acid sequence

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```
    M  K  T  L  L  L  F  V  G  L  L  L  T  W  E  S  G  Q
   -21
             V  L  G  D  Q  T  V  S  D  N  E  L  Q  E  M  S        13
      N  Q  G  S  K  Y  V  N  K  E  I  Q  N  A  V  N  G
      V  K  Q  I  K  T  L  I  E  K  T  N  E  E  R  K           46
   T  L  L  S  N  L  E  E  A  K  K  K  K  E  D  A  L
      N  E  T  R  E  S  E  T  K  L  K  E  L  P  G  V           79
   C  N  E  T  M  M  A  L  W  E  E  C  K  P  C  L  K  Q
         T  C  M  K  F  Y  A  R  V  C  R  S  G  S  G  L       113
      V  G  R  Q  L  E  E  F  L  N  Q  S  S  P  F  Y  F
         W  M  N  G  D  R  I  D  S  L  L  E  N  D  R  Q       146
   Q  T  H  M  L  D  V  M  Q  D  H  F  S  R  A  S  S
      I  I  D  E  L  F  Q  D  R  F  F  T  R  E  P  Q          179
   D  T  Y  H  Y  L  P  F  S  L  P  H  R  R  P  H  F
      F  F  P  K  S  R  I  V  R  S  L  M  P  F  S  P  Y      213
      E  P  L  N  F  H  A  M  F  Q  P  F  L  E  M  I  H
      E  A  Q  Q  A  M  D  I  H  F  H  S  P  A  F  Q         246
   H  P  P  T  E  F  I  R  E  G  D  D  D  R  T  V  C
      R  E  I  R  H  N  S  T  G  C  L  R  M  K  D  Q         279
   C  D  K  C  R  E  I  L  S  V  D  C  S  T  N  N  P
      S  Q  A  K  L  R  R  E  L  D  E  S  L  Q  V  A  E      313
      R  L  T  R  K  Y  N  E  L  L  K  S  Y  Q  W  K  M
      L  N  T  S  S  L  L  E  Q  L  N  E  Q  F  N  W         346
   V  S  R  L  A  N  L  T  Q  G  E  D  Q  Y  Y  L  R
      V  T  T  V  A  S  H  T  S  D  S  D  V  P  S  G         379
   V  T  E  V  V  V  K  L  F  D  S  D  P  I  T  V  T
      V  P  V  E  V  S  R  K  N  P  K  F  M  E  T  V  A      413
      E  K  A  L  Q  E  Y  R  K  K  H  R  E  E  427
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18. A cytolysis inhibitor of claim 15, which is human cytolysis inhibitor.

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19. A cytolysis inhibitor of claim 17, which is glycosylated.

20. A bioactive polypeptide comprising a mutant or fragment of a cytolysis inhibitor of claim 15, which retains the biological activity of the cytolysis inhibitor.

21. A pharmaceutically acceptable salt of a cytolysis inhibitor of claim 15.

22. A pharmaceutical composition comprising a cytolysis inhibitor of claim 15 or a physiologically acceptable salt thereof and a pharmaceutically acceptable excipient.

23. An antibody which specifically binds to a portion of a cytolysis inhibitor protein of claim 15.

24. A monoclonal antibody which specifically binds to a portion of a cytolysis inhibitor protein of claim 15.

25. Monoclonal antibody CLI-9, a monoclonal antibody of claim 24.

26. A hybridoma cell line which produces a monoclonal antibody of claim 24.

27. Hybridoma cell line CLI-9, ECACC No. 89 05 26 02, a hybridoma cell line which produces the monoclonal antibody of claim 25.

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28. A process for producing an isolated cytolysis inhibitor protein having the biological activity of inhibiting cytolysis mediated by a cytolytic protein, comprising expressing a recombinant vector containing a DNA sequence coding for said cytolysis inhibitor protein in a host cell.

29. An isolated recombinant cytolysis inhibitor produced by a process of claim 28.

30. A process for producing an isolated natural cytolysis inhibitor protein having the biological activity of inhibiting cytolysis mediated by a cytolytic protein, comprising subjecting a bodily fluid containing said cytolysis inhibitor protein to affinity chromatography over a solid support to which is attached an affinity agent specific for the cytolysis inhibitor, wherein the affinity agent is a monoclonal antibody of claim 24.

31. A method of treating a condition mediated by cytolytic protein-mediated cell lysis, comprising administering an anti-cytolytically-effective amount of a cytolysis inhibitor of claim 15.

32. A method of claim 31, wherein the condition is inflammation, immunological disorder, infertility, tissue destruction mediated by complement- or perforin-induced cell lysis, or is caused by a membrane-active cytolytic toxin, peptide, and/or protein produced by a pathogenic organism, fungus, or insect.

33. A method of claim 31, wherein the condition is caused by a congenital or acquired deficit of endogenous cytolysis inhibitor or natural CLI plasma component.

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34. A method of inducing a contraceptive effect, comprising administering a vaccine which comprises an amount of an immunogenically effective portion of a cytolysis inhibitor protein of claim 15 and/or the natural carbohydrate portion of said protein, effective to induce an immunogenic response to endogenous cytolysis inhibitor, and thereby effective to induce a contraceptive effect.

35. In a method of detecting and optionally quantitatively determining the presence of a substance in bodily fluids using an immunological molecule, the improvement wherein the substance to be detected and optionally quantitatively determined is a cytolysis inhibitor or a cytolysis inhibitor-containing complex and the immunological molecule is an antibody of claim 23.

36. A method of claim 35, which is a method of diagnosis of a condition or disease wherein changes occur in the amount of cytolysis inhibitor or wherein a cytolysis inhibitor reaction product is produced which retains immunospecificity to said antibody.

37. An isolated CLI-ApoA-I or CLI-HDL complex comprising an isolated natural or recombinant cytolysis inhibitor complexed with ApoA-I, with or without synthetic or natural lipids.

38. A method of treating a condition caused by a disturbance in lipoprotein metabolism or a degenerative vascular condition, comprising administering an effective amount of a cytolysis inhibitor or a CLI-ApoA-I complex or CLI-HDL complex of claim 37.

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39. A method of claim 38, wherein the condition is arteriosclerosis.

40. A method of claim 35, wherein the substance is a cytolysis inhibitor-containing complex comprising CLI-ApoA-I or CLI-HDL, further comprising ApoA-I-specific antibodies.

41. In a method of immunohistological differential diagnosis or in vivo diagnosis involving the determination of the presence and/or location of a substance in bodily tissues using an immunological molecule, the improvement wherein the substance to be determined and/or located is a cytolysis inhibitor or a cytolysis inhibitor-containing complexes and the immunological molecule is a monoclonal antibody of claim 24.

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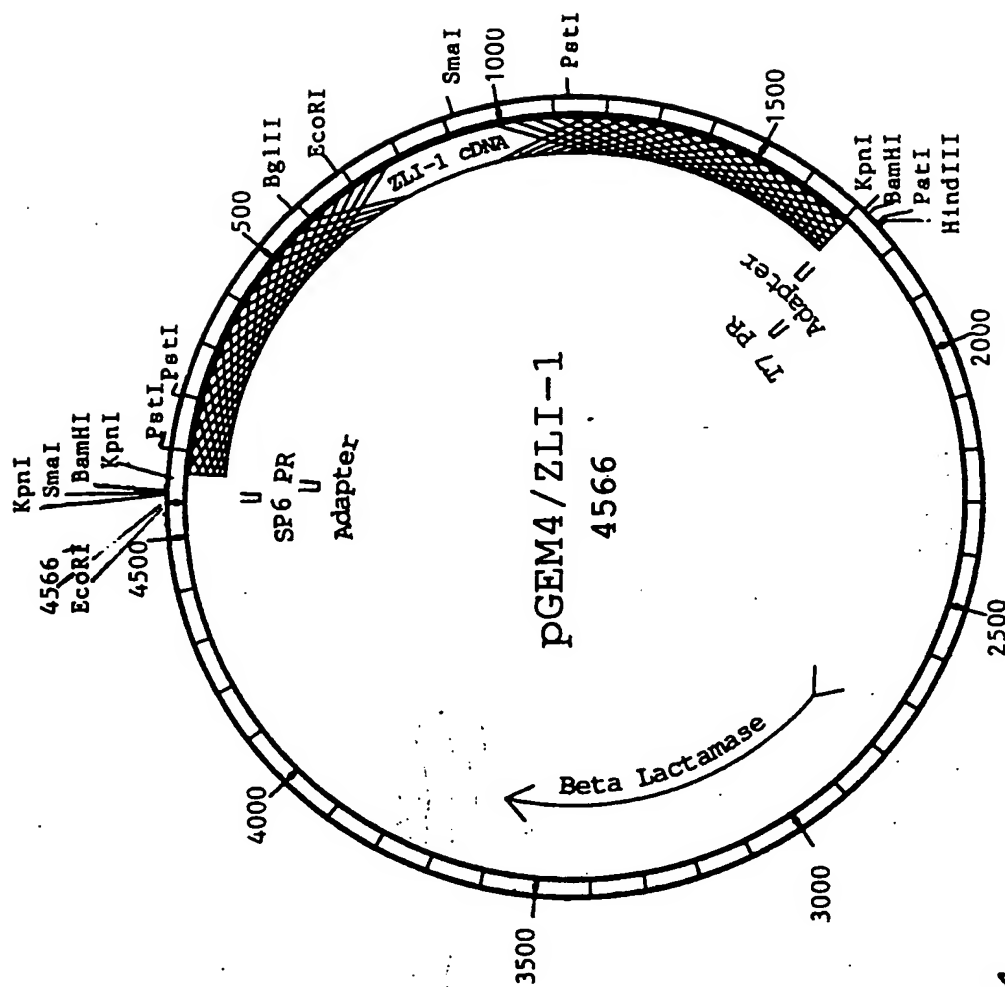
Fig. 1

```
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 99   GGGATGCAGATGGATTCGGTGTGAAGGGCTGGCTGCTGTTGCCTCCGGCTCT
      TGAAAGTCAAGTTCAGAGGCGTGCAAAGACTCCAGAATTGGAGGCATG

199   ATGAAGACTCTGCTGCTGTTTGTGGGGCTGCTGCTGACCTGGGAGAGTGGGC
      M   K   T   L   L   L   F   V   G   L   L   L   T   W   E   S   G   Q
     -21

      AGGTCCTGGGGGACCAGACGGTCTCAGACAATGAGCTCCAGGAAATGT                13
       V   L   G   D   Q   T   V   S   D   N   E   L   Q   E   M   S
                   -1  +1
299   CCAATCAGGGAAGTAAGTACGTCAATAAGGAAATTCAAAATGCTGTCAACGG
       N   Q   G   S   K   Y   V   N   K   E   I   Q   N   A   V   N   G

      GGTGAAACAGATAAAGACTCTCATAGAAAAAAACAAACGAAGAGCGCAA                46
       V   K   Q   I   K   T   L   I   E   K   T   N   E   E   R   K

399   GACACTGCTCAGCAACCTAGAAGAAGCCAAGAAGAAGAAAGAGGATGCCCTA
       T   L   L   S   N   L   E   E   A   K   K   K   E   D   A   L

      AATGAGACCAGGGAATCAGAGACAAAGCTGAAGGAGCTCCCAGGAGTG                79
       N   E   T   R   E   S   E   T   K   L   K   E   L   P   G   V

499   TGCAATGAGACCATGATGGCCCTCTGGGAAGAGTGTAAGCCCTGCCTGAAAC
       C   N   E   T   M   M   A   L   W   E   E   C   K   P   C   L   K   Q

      AGACCTGCATGAAGTTCTACGCACGCGTCTGCAGAAGTGGCTCAGGCC                113
       T   C   M   K   F   Y   A   R   V   C   R   S   G   S   G   L

599   TGGTTGGCCGCCAGCTTGAGGAGTTCCTGAACCAGAGCTCGCCCTTCTACTTC
       V   G   R   Q   L   E   E   F   L   N   Q   S   S   P   F   Y   F

      TGGATGAATGGTGACCGCATCGACTCCCTGCTGGAGAACGACCGGCA                146
       W   M   N   G   D   R   I   D   S   L   L   E   N   D   R   Q

699   GCAGACGCACATGCTGGATGTCATGCAGGACCACTTCAGCCGCGCGTCCAGC
       Q   T   H   M   L   D   V   M   Q   D   H   F   S   R   A   S   S

      ATCATAGACGAGCTCTTCCAGGACAGGTTCTTCACCCGGGAGCCCCAG                179
       I   I   D   E   L   F   Q   D   R   F   F   T   R   E   P   Q

799   GATACCTACCACTACCTGCCCTTCAGCCTGCCCCACCGGAGGCCTCACTTC
       D   T   Y   H   Y   L   P   F   S   L   P   H   R   R   P   H   F

      TTCTTTCCCAAGTCCCGCATCGTCCGCAGCTTGATGCCCTTCTCTCCGT                213
       F   F   P   K   S   R   I   V   R   S   L   M   P   F   S   P   Y

899   ACGAGCCCCTGAACTTCCACGCCATGTTCCAGCCCTTCCTTGAGATGATAC
       E   P   L   N   F   H   A   M   F   Q   P   F   L   E   M   I   H

      ACGAGGCTCAGCAGGCCATGGACATCCACTTCCACAGCCCGGCCTTCCA                246
       E   A   Q   Q   A   M   D   I   H   F   H   S   P   A   F   Q

999   GCACCCGCCAACAGAATTCATACGAGAAGGCGACGATGACCGGACTGTGTG
       H   P   P   T   E   F   I   R   E   G   D   D   D   R   T   V   C
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      CCGGGAGATCCGCCACAACTCCACGGGCTGCCTGCGGATGAAGGACCAG
       R  E  I  R  H  N  S  T  G  C  L  R  M  K  D  Q        279

 1099 TGTGACAAGTGCCGGGAGATCTTGTCTGTGGACTGTTCCACCAACAACCCC
       C  D  K  C  R  E  I  L  S  V  D  C  S  T  N  N  P

      TCCCAGGCTAAGCTGCGGCGGGAGCTCGACGAATCCCTCCAGGTCGCTG
     · S  Q  A  K  L  R  R  E  L  D  E  S  L  Q  V  A  E    313

 1199 AGAGGTTGACCAGGAAATATAACGAGCTGCTAAAGTCCTACCAGTGGAAGA
       R  L  T  R  K  Y  N  E  L  L  K  S  Y  Q  W  K  M

      TGCTCAACACCTCCTCCTTGCTGGAGCAGCTGAACGAGCAGTTTAACTG
       L  N  T  S  S  L  L  E  Q  L  N  E  Q  F  N  W      346

 1299 GGTGTCCCGGCTGGCAAACCTCACGCAAGGCGAAGACCAGTACTATCTGCG
       V  S  R  L  A  N  L  T  Q  G  E  D  Q  Y  Y  L  R

      GGTCACCACGGTGGCTTCCCACACTTCTGACTCGGACGTTCCTTCCGGT
       V  T  T  V  A  S  H  T  S  D  S  D  V  P  S  G     379

 1399 GTCACTGAGGTGGTCGTGAAGCTCTTTGACTCTGATCCCATCACTGTGACG
       V  T  E  V  V  V  K  L  F  D  S  D  P  I  T  V  T

      GTCCCTGTAGAAGTCTCCAGGAAGAACCCTAAATTTATGGAGACCGTGG
       V  P  V  E  V  S  R  K  N  P  K  F  M  E  T  V  A    413

 1499 CGGAGAAAGCGCTGCAGGAATACCGCAAAAAGCACCGGGAGGAGTGAGATG
       E  K  A  L  Q  E  Y  R  K  K  H  R  E  E  427

      TGGATGTTGCTTTTGCACCTACGGGGGCATCTGAGTCCAGCTCCCCCCA

 1599 AGATGAGCTGCAGCCCCCCAGAGAGAGCTCTGCACGTCACCAAGTAACCAGGC   1651
```

Fig. 2

Fig. 3

66—

45—

nr   r

Fig. 4

Fig. 4b

97 —

68 —

45 —

29 —
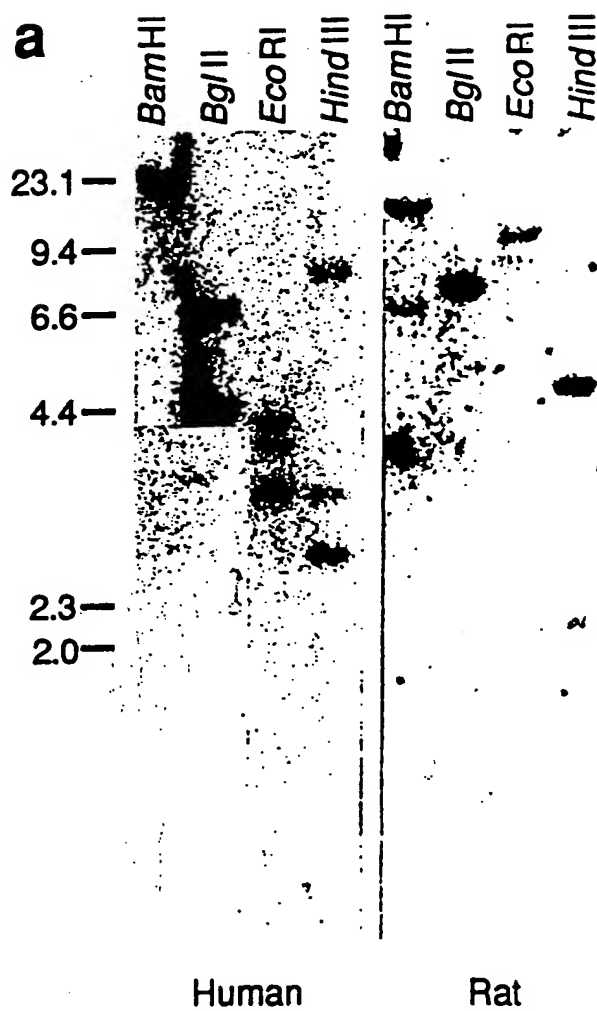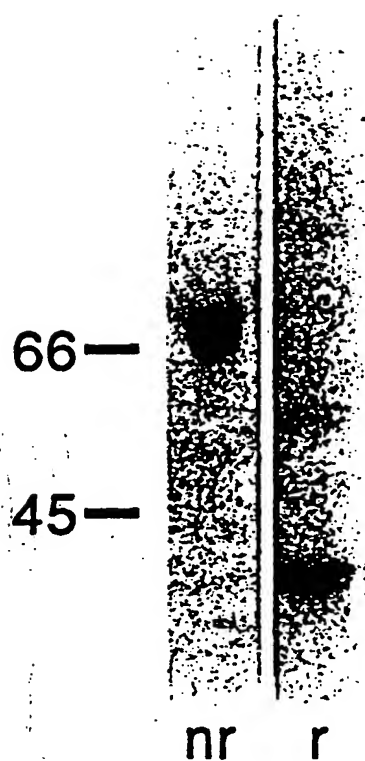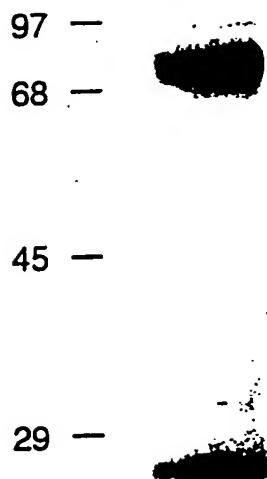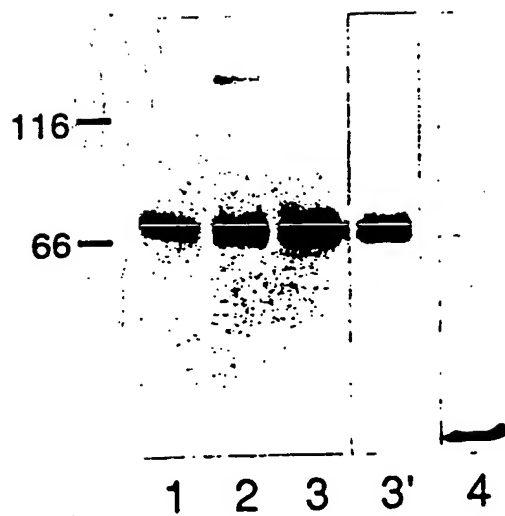
Fig. 5

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INHIBITION OF COMPLEMENT CYTOLYSIS
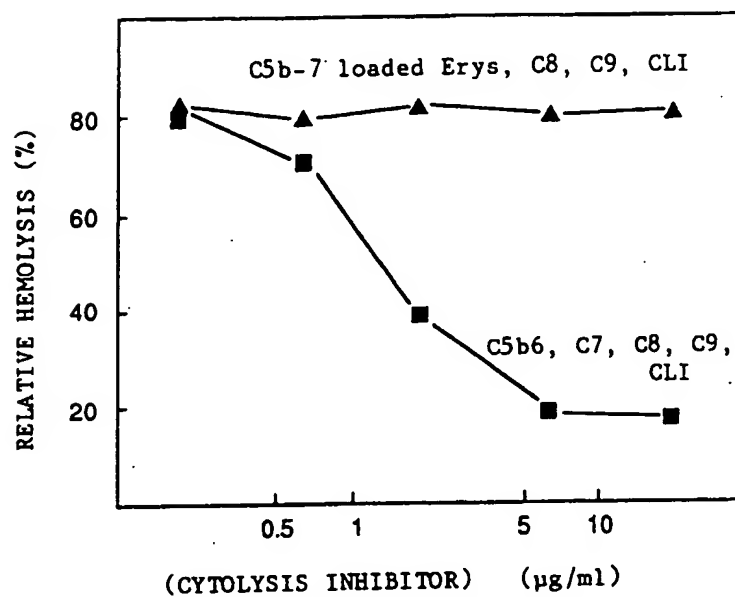
Fig. 6

INHIBITION OF CTL/NK PERFORIN CYTOLYSIS
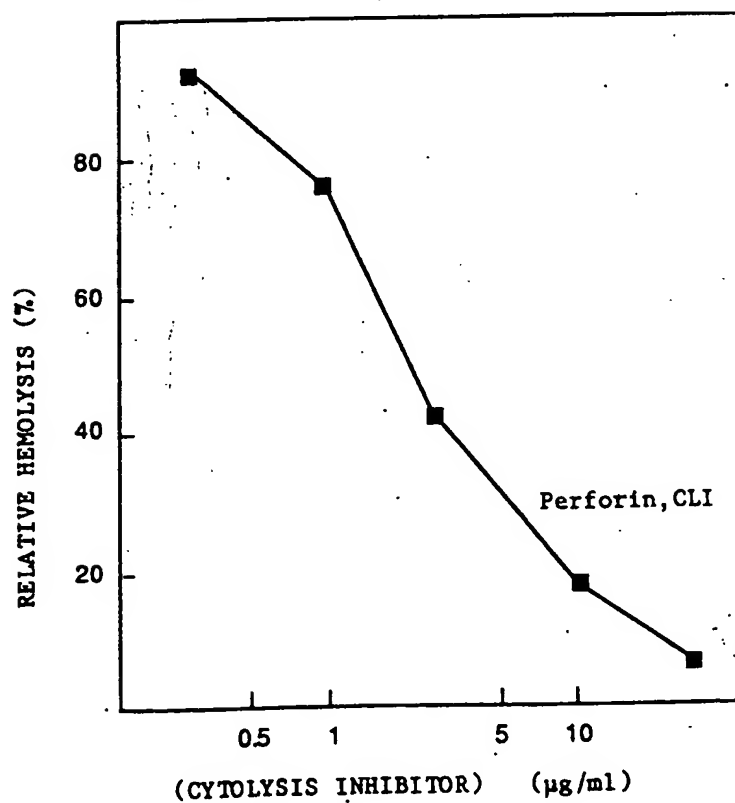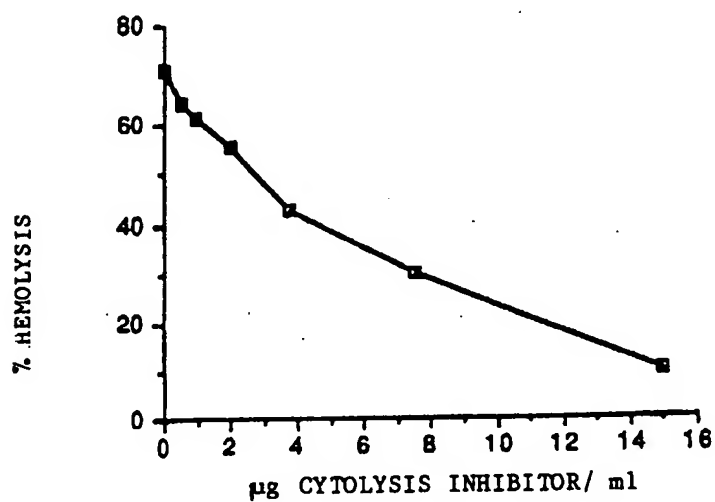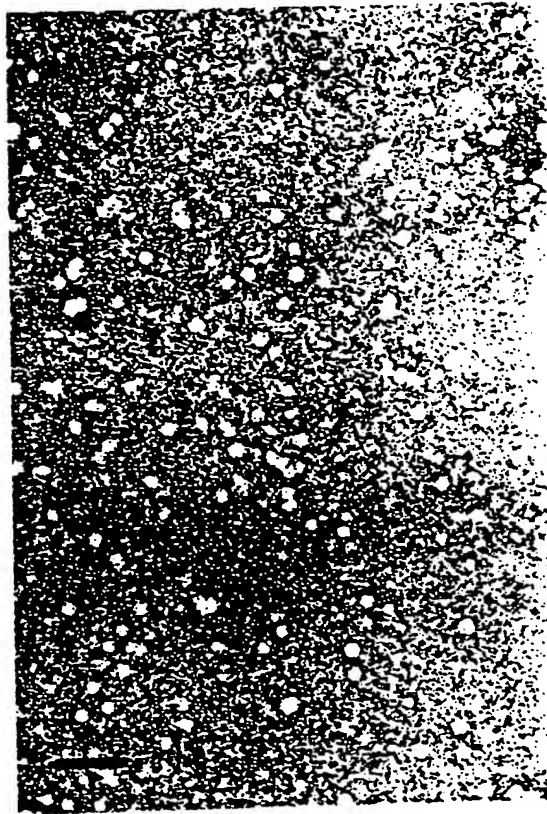
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*Fig. 7*

Fig. 8

Fig. 9

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5 : C 12 N 15/12, 1/21, C 12 P 21/02, A 61 K 37/02,
C 12 P 21/08, G 01 N 33/68, C 07 K 13/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Proc. Natl. Acad. Sci. USA, volume 86, September 1989, (Washington, DC, US), D.E. Jenne et al.: "Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: Identity to sulfated glycoprotein 2, a constituent of rat testis fluid", pages 7123-7127 see the whole article --	1-30,35
X	The EMBO Journal, volume 8, no. 3, March 1989, IRL Press, (Oxford, GB), L. Kirszbaum et al.: "Molecular cloning and characterization of the novel, human complement-associated protein, SP-40,40: a link between the complement and reproductive systems", pages 711-718 see the whole article -- ./.	1-30,35

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th December 1990	2 2. 01. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Natalie Weinberg

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,X	Biochemistry, volume 29, no. 22, 1990, American Chemical Society, H.V. de Silva et al.: "Apolipoprotein J: Structure and tissue distribution", pages 5380-5389 see the whole article	1-30,35,37

V.☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☒ Claim numbers __**__, because they relate to subject matter not required to be searched by this Authority, namely:

   ** Unsearchable Claims 31-34, 38,39
     Partly unsearchable claims 41 (only as far as it concerns no invivo diagnosis)
    See Rule 39.1 (iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods,

2.☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This international Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

  ☐ The additional search fees were accompanied by applicant's protest.

  ☐ No protest accompanied the payment of additional search fees.